Further characterization of the platinum-reactive component of the α_2 -macroglobulin-receptor recognition site

Salvatore V. PIZZO, Paul A. ROCHE, Steven R. FELDMAN and Steven L. GONIAS

Departments of Pathology and Biochemistry, Duke University Medical Center, Durham, NC 27710, U.S.A.

α_2 -Macroglobulin (α_2 M)-methylamine that had been allowed to react with *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) bound with greatly reduced affinity to specific α_2 M receptors, as determined by macrophage binding studies *in vitro* and plasma-clearance experiments *in vivo*. Subsequent reaction with diethyl dithiocarbamate completely restored receptor recognition function. The optimal effect was obtained when the diethyl dithiocarbamate concentration was twice the total platinum concentration. α_2 M-methylamine that was allowed to react with H_2O_2 competed less effectively for specific cell-surface binding sites, as demonstrated by studies both *in vivo* and *in vitro*. The apparent dissociation constant was increased nearly 7-fold by a 15 min exposure to H_2O_2 . α_2 M-methylamine was affected significantly less by the H_2O_2 exposure after pretreatment with *cis*-DDP. Amino acid analysis indicated that H_2O_2 treatment of α_2 M modified 19 of the 25 methionine residues per α_2 M subunit. Pretreatment with *cis*-DDP protected two to four of these methionine residues. The only other residue altered by H_2O_2 treatment of α_2 M was histidine. A net decrease of two histidine residues per subunit was observed, but *cis*-DDP pretreatment did not alter this result. In order to rule out the slight possibility that histidine modification might account for the observed H_2O_2 -induced loss in receptor recognition, diethyl pyrocarbonate was employed as a histidine-modifying reagent. This treatment modified 53 histidine residues in both native and fast-form α_2 M. Fast-form α_2 M was still recognized by the α_2 M receptor, as determined by studies both *in vivo* and *in vitro*; however, a fraction of the modified protein now cleared via the acyl-low-density-lipoprotein receptor as well. Reaction of diethyl pyrocarbonate-treated α_2 M with hydroxylamine reversed derivatization of 43 of the 53 histidine residues. Moreover, this treatment also resulted in an α_2 M fast-form preparation that was recognized only by the α_2 M receptor. It is concluded that *cis*-DDP and H_2O_2 modify a critical methionine residue in the primary sequence of the α_2 M-receptor recognition site.

INTRODUCTION

Human α_2 -macroglobulin (α_2 M) is a high- M_r plasma proteinase inhibitor of known primary structure (Sottrup-Jensen *et al.*, 1984). Reaction of α_2 M with a broad range of functionally different endopeptidases results in a conformational change in the proteinase inhibitor (Barrett *et al.*, 1979; Björk & Fish, 1982; Gonias *et al.*, 1982) with 'trapping' of the proteinase (Barrett & Starkey, 1973; Barrett *et al.*, 1979; Salvesen *et al.*, 1981). An equivalent conformational change occurs after reaction with primary amines (Barrett *et al.*, 1979; Björk & Fish, 1982; Gonias *et al.*, 1982). Only the structural form of α_2 M that has reacted with proteinases or amines ('fast' form) is recognized by specific membrane receptors on hepatocytes, adipocytes, fibroblasts and reticuloendothelial cells (Van Leuven *et al.*, 1979; Imber & Pizzo, 1981; Gliemann *et al.*, 1983; Ney *et al.*, 1984; Pizzo & Gonias, 1984; Feldman *et al.*, 1985b). The sequence of α_2 M amino acids recognized by the receptor remains undefined. Carbohydrate residues are not critical in the process (Imber & Pizzo, 1981).

Reaction of α_2 M-methylamine with 2,4,6-trinitrobenzenesulphonate abolishes receptor-mediated endocytosis of the derivative as well as reaction with monoclonal antibodies directed towards the receptor

recognition site (Marynen *et al.*, 1982). These data provide evidence that lysine may be a necessary residue in the recognized sequence. Receptor-mediated plasma clearance of α_2 M-methylamine and α_2 M-proteinase complex is significantly inhibited by their reaction with low concentrations of the anti-tumour drug *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) (Gonias & Pizzo, 1981a). When native α_2 M is allowed to react with *cis*-DDP before treatment with methylamine, receptor recognition is not affected to as great an extent. This suggests that platinum binds specifically to the receptor recognition site, which is exposed by conformational change (Gonias & Pizzo, 1983a). The *cis*-DDP-reactive sites on α_2 M have not been characterized; however, a number of studies indicate that the reaction of *cis*-DDP with proteins is specific (Gonias & Pizzo, 1983a; Aull *et al.*, 1977). Serum albumin binds 1–2 g-atom of platinum per mol of protein maximally, and the major fraction of this binding reflects reaction with a single residue, namely cysteine-34 (Gonias & Pizzo, 1983a). In nearly all characterized systems, cysteine and methionine residues serve as the platinum-binding sites in proteins (Dickerson *et al.*, 1969; Howe-Grant & Lippard, 1980; Gonias *et al.*, 1984). The primary structure of α_2 M-methylamine includes a single cysteine residue in each of four subunits that is generated by cleavage of a thioester bond

Abbreviations used: *cis*-DDP, *cis*-dichlorodiammineplatinum(II); α_2 M, α_2 -macroglobulin; LDL, low-density lipoprotein; BSA, bovine serum albumin.

(Swenson & Howard, 1979; Sottrup-Jensen *et al.*, 1980; Salvesen *et al.*, 1981). The cysteine residues are not involved in receptor recognition (Gonias & Pizzo, 1981a).

In the present investigation macrophage binding experiments *in vitro* with *cis*-DDP-treated α_2 M are presented for the first time in conjunction with studies *in vivo*. The reagent diethyl dithiocarbamate was used to demonstrate that receptor recognition may be fully restored to modified α_2 M by removal of bound platinum. Treatment of α_2 M fast-form with H_2O_2 also results in loss of receptor recognition. Platinum is shown to protect significantly the receptor-recognition function of α_2 M in the presence of H_2O_2 , supporting the importance of methionine in receptor binding. Amino acid analysis documents modification of 19 out of 25 methionine residues per α_2 M subunit, and *cis*-DPP pretreatment protects two to four of these residues.

EXPERIMENTAL PROCEDURES

Materials

Human α_2 M and α_2 M-methylamine were prepared as previously described (Imber & Pizzo, 1981). Carrier-free $Na^{125}I$ was obtained from New England Nuclear. Radioiodination of α_2 M was performed by the method of David & Reisfeld (1974). *cis*-DDP, diethyl pyrocarbonate and diethyl dithiocarbamate were purchased from Aldrich. Maleylated BSA was prepared and characterized as previously described (Imber *et al.*, 1982; Johnson *et al.*, 1982). Macroalbumin was prepared and characterized as described by Iio *et al.* (1963). CD-1 female mice (20 weeks old) were purchased from Charles River Laboratories, Wilmington, MA, U.S.A. Thioglycollate-stimulated mouse peritoneal macrophages were isolated as previously described (Adams, 1979).

Chemical modification of α_2 M with *cis*-DDP and H_2O_2

^{125}I - α_2 M-methylamine (0.14 μ M) and 1.7 mM-*cis*-DDP were incubated for 4 h at 37 °C in 20 mM-Tris/HCl/100 mM-NaCl, pH 7.4. The preparation was divided into several portions, which were allowed to react with various concentrations of diethyl dithiocarbamate at 37 °C for 6 h. Diethyl dithiocarbamate-platinum complexes are insoluble under standard aqueous conditions (Gonias *et al.*, 1984) and the precipitates that formed were removed by a short period of centrifugation in an Eppendorf microcentrifuge.

^{125}I - α_2 M-methylamine was allowed to react with 6% (v/v) H_2O_2 for 15 min in 20 mM-Tris/HCl/100 mM-NaCl, pH 7.4. Excess catalase was added to stop the reaction. Since treatment of ^{125}I - α_2 M-methylamine with H_2O_2 was found to result in a variable loss of the label from the tyrosine residues, the studies were also performed with α_2 M treated first with H_2O_2 and then radiolabelled by the method of David & Reisfeld (1974). Equivalent results were obtained with either preparation in all the studies to be described here.

In the final series of studies, α_2 M-methylamine was allowed to react with 1.7 mM-*cis*-DDP for 4 h and then 6% (v/v) H_2O_2 for 15 min, followed by catalase. The solution was made 3.5 mM in diethyl dithiocarbamate for 6 h at 37 °C to remove the platinum. The precipitate was removed as described above.

Chemical modification of α_2 M by diethyl pyrocarbonate and reversal of histidine modification by hydroxylamine

α_2 M, α_2 M-methylamine, and α_2 M-trypsin (0.14 μ M) were incubated with various concentrations of diethyl pyrocarbonate (dissolved in 100% ethanol) for 5 min at room temperature (Miles, 1978). The extent of histidine modification was determined by u.v. difference spectroscopy over the range 200–300 nm in a Shimadzu u.v.-visible recording spectrophotometer (Model UV-240). Since lysine residues may also be modified by this reagent, particularly at high concentrations (Miles, 1978), reversal experiments were performed to probe this chemical modification. Hydroxylamine treatment of diethyl pyrocarbonate-modified proteins removes the carbethoxy group from modified histidine, but not lysine, residues. For the reversal experiments, hydroxylamine hydrochloride was prepared as a stock solution in sodium phosphate-buffered saline, pH 7.4. α_2 M-methylamine, pretreated with 1 mM-diethyl pyrocarbonate, was incubated with hydroxylamine at a final concentration of 250 mM for 5 min. U.v. difference spectra were then recorded 15 min, 1 h and 2 h after addition. In one series of experiments, α_2 M fast-form (0.14 μ M) was modified with *cis*-DDP as described above and then treated with various concentrations of diethyl pyrocarbonate for 5 min at room temperature.

α_2 M preparations were labelled with ^{125}I by the method of David & Reisfeld (1974) for use in clearance studies in mice and binding studies *in vitro* with macrophages.

Amino acid analysis

Amino acid analysis was performed on a Beckman 6300 high-performance amino acid analyser. Preparations of α_2 M treated with H_2O_2 alone or *cis*-DDP followed by H_2O_2 were hydrolysed *in vacuo* for 24 h at 110 °C in 6 M-HCl before analysis.

Cell binding experiments *in vitro*

The binding of unlabelled and modified α_2 M preparations to plated macrophages at 4 °C was studied in reversible competition experiments with ^{125}I - α_2 M-methylamine. The concentration of ^{125}I - α_2 M-methylamine was standardized at 0.3 nM. The concentration of the unlabelled α_2 M preparation was varied. Cell preparation, cell media, buffers and incubation conditions were as previously described (Imber & Pizzo, 1981). After equilibration, cells were dissolved in 0.1 M-NaOH and harvested. Cell-bound radioactivity was measured in a Scientific Product AW-14120 γ -radiation counter. The concentration of unlabelled ligand required to decrease the binding of ^{125}I - α_2 M-methylamine to cells by 50% was designated the ' I_{50} '. The I_{50} is related to the apparent dissociation constant of the non-radiolabelled protein (K_I) by the equation:

$$I_{50} = K_I [1 + (L/K_L)]$$

where L and K_L are the concentration and dissociation constant for radioiodinated α_2 M-methylamine respectively. All experiments were performed in triplicate and the results averaged.

Plasma-clearance experiments *in vivo*

Preparations to be studied were injected into the lateral tail veins of CD-1 female mice. Clearance was

Fig. 1. Receptor binding of radiolabelled α_2 M fast-form *in vitro*: competition by α_2 M fast-form-platinum complex

α_2 M-methylamine (0.14 μ M) was allowed to react with *cis*-DDP for 4 h at 37 °C in 20 mM-Tris/HCl/100 mM-NaCl, pH 7.4. The *cis*-DDP concentration was 0.8 mM ($\diamond$), 1.2 mM ($\circ$) or 1.7 mM ($\square$). Each preparation was then incubated with plated macrophages for 8 h in the presence of radiolabelled- α_2 M-methylamine (0.3 mM). The abscissa specifies the concentration of the *cis*-DDP-treated protein in the cell incubation medium. The inhibition of radioligand binding by unlabelled- α_2 M-methylamine that was *not* allowed to react with *cis*-DDP is shown for comparison ($\triangle$). Conditions for the binding studies have been reported previously (Imber & Pizzo, 1981). In brief, binding studies were performed at 4 °C with fixed monolayers of thioglycollate-elicited murine peritoneal macrophages plated at a density of 200 000 cells/well. After the cells had been incubated with radioligand and unlabelled ligand for up to 3 h, the cells are extensively washed at 4 °C and solubilized in NaOH for radioactivity counting in a γ -radiation counter. All data are normalized for variation in cell numbers by determining the total protein content.

Fig. 2. Plasma clearance of radioiodinated α_2 M fast-form after reaction with *cis*-DDP and diethyl dithiocarbamate

125 I- α_2 M-methylamine was allowed to react with 1.7 mM-*cis*-DDP under conditions specified in the Experimental procedures section. The protein was then incubated with diethyl dithiocarbamate at the following concentrations: 0 mM ($\square$); 1.0 mM ($\diamond$); 3.5 mM (∇); 7.0 mM ($\bullet$). The clearances of 125 I- α_2 M ($\circ$) and 125 I- α_2 M-methylamine ($\triangle$) are shown for comparison. In each experiment, 1 μ g of radiolabelled protein was injected.

followed by repetitive blood sampling from the retro-orbital venous plexus. The procedure is described in detail elsewhere (Imber & Pizzo, 1981; Gonias *et al.*, 1984). All studies were performed, on average, four times.

RESULTS

Binding studies *in vitro* with *cis*-DDP-treated α_2 M

Unlabelled α_2 M-methylamine inhibited the binding of 125 I- α_2 M-methylamine to macrophages in a concentration-dependent manner (Fig. 1) with an I_{50} of 0.6 nM. α_2 M-methylamine that was treated with *cis*-DDP (0.8–1.7 mM) inhibited radioligand binding only at significantly higher concentrations. α_2 M-methylamine allowed to react with 1.7 mM-*cis*-DDP was least effective at competing for membrane binding sites and the I_{50} was 22 nM. Identical results were obtained in experiments in which dialysis or gel-filtration chromatography was utilized to separate unbound *cis*-DDP from the α_2 M-methylamine before incubation with cells. These data are in good agreement with those obtained in previously presented clearance experiments utilizing similarly prepared α_2 M-*cis*-DDP complexes (Gonias & Pizzo, 1981a).

Reversibility of *cis*-DDP-induced receptor recognition inhibition

In standard aqueous buffers, under physiological conditions, the reaction of α_2 M with *cis*-DDP is essentially irreversible (Gonias & Pizzo, 1981a,b). Diethyl dithiocarbamate is a potent nucleophile that can displace ligands from platinum-binding sites even when these bonds are extremely stable (Gonias *et al.*, 1984). Fig. 2 shows the effect of diethyl dithiocarbamate on the plasma clearance of α_2 M-methylamine that had been allowed to react with *cis*-DDP. α_2 M-methylamine cleared rapidly from the circulation of mice, reflecting binding and endocytosis by specific membrane receptors, as previously described (Gonias & Pizzo, 1983b). *cis*-DDP-treated protein cleared at a rate that was only slightly higher than that of native α_2 M, which is not recognized by the receptor. Inhibition of clearance was reversed by a period of incubation with diethyl dithiocarbamate at concentrations at least 2-fold the total platinum concentration. The stable platinum complex formed during this reaction probably contains 2 mol of diethyl dithiocarbamate/mol of platinum (Gonias *et al.*, 1984), accounting for the observed concentration-dependency. The ability of diethyl dithiocarbamate to restore completely receptor recognition

Fig. 3. Receptor binding by radiolabelled α_2 M-methylamine *in vitro*: competition by α_2 M-methylamine that had been allowed to react with H_2O_2 and *cis*-DDP

α_2 M-methylamine was allowed to react with 1.7 mM-*cis*-DDP for 4 h at 37 °C. Diethyl dithiocarbamate was then added to the solution to a final concentration of 3.5 mM for 6 h at 37 °C and the preparation was transferred to a cell binding plate at 4 °C ($\diamond$). α_2 M-methylamine was allowed to react with 6% (v/v) H_2O_2 for 15 min in 20 mM-Tris/HCl/100 mM-NaCl, pH 7.4. Excess catalase was added to stop the reaction before transferring the solution to a cell binding plate ($\bullet$). α_2 M-methylamine was allowed to react with 1.7 mM-*cis*-DDP for 4 h and then with 6% (v/v) H_2O_2 for 15 min, followed by catalase. The solution was then made 3.5 mM in diethyl dithiocarbamate for 6 h at 37 °C (Δ). α_2 M-methylamine that was not treated with any of these reagents is shown for comparison ($\square$). The abscissa specifies the concentration of non-radioiodinated α_2 M in the cell incubation medium.

function to *cis*-DDP-treated α_2 M-methylamine was confirmed in the macrophage binding system *in vitro* (Fig. 3).

H_2O_2 reactions

α_2 M-methylamine was incubated with 6% (v/v) H_2O_2 for 15 min before addition of catalase to stop the reaction. The clearance of this preparation is shown in Fig. 4. Reaction of α_2 M fast-form with H_2O_2 resulted in a decrease in the rate of protein clearance. In experiments *in vitro*, peroxide-treated α_2 M-methylamine competed effectively for binding sites only at significantly higher concentrations than the control preparation of α_2 M-methylamine (Fig. 3). The K_I for H_2O_2 -treated α_2 M-methylamine was 2 nM, nearly 7 times that for untreated α_2 M-methylamine. Assuming either complete loss or retention of receptor binding activity by the H_2O_2 -treated protein, 15% of the α_2 M-methylamine retained activity.

α_2 M-methylamine was allowed to react with *cis*-DDP before exposure to H_2O_2 under conditions equivalent to those described above. The H_2O_2 was inactivated with catalase and the preparation was then treated with diethyl dithiocarbamate. The resulting preparation inhibited the binding of ^{125}I - α_2 M-methylamine to cells less effectively than the binding of unmodified α_2 M-methylamine, reflecting reaction with H_2O_2 . However, the decrease in receptor binding activity caused by H_2O_2 was of significantly lower magnitude after pretreatment with *cis*-DDP. Assuming complete loss or retention of

Fig. 4. Plasma clearance of radioiodinated α_2 M-methylamine after reaction with H_2O_2

The preparation was allowed to react with 6% (v/v) H_2O_2 for 15 min in 20 mM-Tris/HCl/100 mM-NaCl, pH 7.4. Excess catalase was added to stop the reaction before clearance. Symbols: Δ , α_2 M-methylamine; $\blacktriangle$, H_2O_2 -treated α_2 M-methylamine. In each experiment, 1 μ g of radiolabelled protein was injected.

Table 1. Summary of data from assays of receptor binding by radiolabelled α_2 M

Results are taken from Figs. 1 and 3. Abbreviation used: DDC, diethyl dithiocarbamate.

α_2 M-methylamine	I_{50} (nM)	K_I (nM)	Receptor- reactive* (%)
Untreated	0.6	0.3	100
Treated with <i>cis</i> -DDP at:			
0.8 mM	10	5	6
1.2 mM	16	8	4
1.7 mM	22	11	3
1.7 mM- <i>cis</i> -DDP + 3.5 mM-DDC	0.6	0.3	100
H_2O_2	4	2	15
<i>cis</i> -DDP + H_2O_2 + DDC	1	0.5	60

* As determined by binding studies *in vitro*, where α_2 M-methylamine is assumed to be 100% receptor-reactive.

receptor binding activity as described above, 60% of α_2 M-methylamine retained binding activity after sequential reaction with *cis*-DDP, H_2O_2 and diethyl dithiocarbamate. These data indicate that *cis*-DDP inhibits the reaction of H_2O_2 with a critical amino acid at the

receptor recognition site, preventing the modification that renders the protein unreactive towards the receptor. The data are summarized in Table 1.

Amino acid analysis

Amino acid analysis was employed to characterize further the reaction of α_2 M-methylamine with H_2O_2 in the presence or absence of *cis*-DDP. These studies demonstrated that approx. 19 of 25 methionine residues per α_2 M subunit were oxidized by H_2O_2 . In addition, two of the 39 histidine residues per α_2 M subunit were lost after treatment of α_2 M-methylamine with H_2O_2 . *cis*-DDP pretreatment of α_2 M-methylamine protected between two and four of the 19 methionine residues per α_2 M subunit, but did not prevent oxidation of the two histidine residues. Also of importance, H_2O_2 did not oxidize any other amino acids in α_2 M-methylamine. These data are consistent with the presence of a methionine residue at the α_2 M-fast-form-receptor recognition site.

Histidine residues in the receptor recognition of α_2 M fast-forms

cis-DDP reacts with histidine residues, albeit at a much lower rate than with methionine (Gonias *et al.*, 1984). Since two residues of histidine were modified by H_2O_2 per α_2 M subunit, it was decided to study further the potential role of histidine residues in the receptor recognition of α_2 M fast-form. α_2 M, α_2 M-methylamine and α_2 M-trypsin were allowed to react with 1 mM-diethyl pyrocarbonate. The extent of histidine modification was determined by u.v. difference spectroscopy (Fig. 5). In both native and fast-form α_2 M, 53 of 156 histidine residues were modified per molecule. The extent of modification was constant when α_2 M concentration was varied from 7.0 nM to 0.14 μ M (results not shown). When α_2 M-methylamine was treated with 1 mM-diethyl pyrocarbonate and hydroxylamine in sequence, 43 of the 53 modified histidines were regenerated. Failure to reverse the modification of the remaining ten residues probably reflects two-point modification of particularly susceptible histidine residues (Miles, 1978).

The effect of histidine modification on the clearance of native and α_2 M fast-form was studied (Figs. 6 and 7). The rate of clearance of α_2 M fast-form treated with various concentrations of diethyl pyrocarbonate was very rapid (Fig. 6). Clearance half-lives progressively shortened with increasing concentrations of diethyl pyrocarbonate. The α_2 M fast-form preparation treated with 1 mM-diethyl pyrocarbonate demonstrated a half-life of only 30 s. This effect was observed with both α_2 M-methylamine and α_2 M-trypsin. Co-injection of the α_2 M fast-form with 2 mg of macroalbumin did not alter the clearance of the inhibitor. This result indicates that the rapid clearance did not result from denaturation of the α_2 M resulting in non-specific reticuloendothelial clearance.

The clearance of the α_2 M fast-form treated with 50 μ M-diethyl pyrocarbonate was blocked by competition with a 1000-fold molar excess of unmodified α_2 M fast-form (Fig. 6a). The clearance of α_2 M fast-form treated with 1 mM-diethyl pyrocarbonate was not inhibited by a similar dose of competing ligand (Fig. 6c). An intermediate degree of competition was demonstrated with α_2 M fast-form treated with 250 μ M-diethyl pyrocarbonate.

Fig. 5. U.v. difference spectra comparing α_2 M before and after reaction with diethyl pyrocarbonate

Measurements were made in a split-beam instrument with tandem (two-chamber) cuvettes. (a) The sample cuvette contained native α_2 M (0.14 μ M) that had been allowed to react with 1 mM-diethyl pyrocarbonate in one chamber and buffer only in the second chamber. The reference cuvette contained native α_2 M (0.14 μ M) in the first chamber and 1 mM-diethyl pyrocarbonate in the adjacent chamber. (b) Three separate difference spectra are shown. The first spectrum (—) compares α_2 M-methylamine (0.14 μ M) that had been allowed to react with 1 mM-diethyl pyrocarbonate in the sample cuvette with the protein and reagent in adjacent chambers of the reference cuvette. Hydroxylamine (250 mM) was then added to the chamber that contained protein in the sample cuvette and the chamber that contained diethyl pyrocarbonate in the reference cuvette. Different spectra were remeasured after 15 min (----) and 1 h (○). An additional spectrum measured after 2 h remained equivalent to the 1 h spectrum and is not shown. Control experiments demonstrated that hydroxylamine has no effect on the u.v. spectrum of α_2 M-methylamine under the conditions employed in these studies.

One possible explanation of the results from these clearance studies is that the diethyl pyrocarbonate modified lysine residues and/or histidine residues yielded a product recognized by the acyl-low-density-lipoprotein (LDL) receptor (Goldstein *et al.*, 1979a,b). A test for clearance via the acyl-LDL receptor involves the use of maleylated BSA as a competing ligand. Co-injection of maleylated BSA (2 mg) with 125 I- α_2 M fast-form treated with diethyl pyrocarbonate did not prevent clearance. When the modified 125 I- α_2 M fast form was injected in the presence of both a 1000-fold excess of α_2 M fast-form and

Fig. 6. Plasma clearance of radioiodinated α_2 M fast-form after reaction with diethyl pyrocarbonate

(a) α_2 M fast-form that had been allowed to react with 50 μ M-diethyl pyrocarbonate for 5 min and injected alone (●) or with a 1000-fold excess of α_2 M-methylamine (□). (b) α_2 M fast-form that had been allowed to react with 250 μ M-diethyl pyrocarbonate for 5 min and injected alone (●), with 2 mg of maleylated BSA (△), a 1000-fold excess of α_2 M-methylamine (□) or a combination of both competing ligands (▲). (c) α_2 M fast-form that had been allowed to react with 1 mM-diethyl pyrocarbonate for 5 min and injected alone (●), with 2 mg of macroalbumin (○), with 2 mg of maleylated BSA (△), a 1000-fold excess of α_2 M-methylamine (□) or a combination of both competing ligands (▲), treated with 250 μ M-hydroxylamine for 15 min after reaction with 1 mM-diethyl pyrocarbonate and injected alone (●) or with 1000-fold molar excess of α_2 M-methylamine (■). In each experiment, 1 μ g of radiolabelled protein was injected.

2 mg of maleylated BSA, marked competition for clearance was observed, supporting the conclusion that diethyl pyrocarbonate-modified α_2 M fast-form clears via the α_2 M fast-form receptor and/or via the acyl-LDL receptor.

Since previous studies have indicated that modification of lysine residues is responsible for recognition of acylated proteins (Goldstein *et al.*, 1979a,b), we decided to probe further the reaction of diethyl pyrocarbonate with α_2 M fast-forms. Hydroxylamine reverses monocarboxylation of histidine, but does not react with histidine residues that have undergone dicarboxylation or modified lysine residues (Miles, 1978). We employed hydroxylamine treatment as described above (Fig. 5) to reverse the derivatization of α_2 M fast-form treated with 1 mM-diethyl pyrocarbonate. This reaction

Fig. 7. Plasma clearance of radioiodinated α_2 M after reaction with diethyl pyrocarbonate

α_2 M was allowed to react with 1 mM-diethyl pyrocarbonate for 5 min and injected into mice (■). The same preparation was injected after reaction with trypsin (●). The preparation treated with trypsin was then co-injected with a 1000-fold excess of α_2 M-methylamine (□), 2 mg of maleylated-BSA (△) or a combination of both competing ligands (▲). The preparation of α_2 M that had been allowed to react with diethyl pyrocarbonate was then treated with 250 mM-hydroxylamine for 15 min, allowed to react with trypsin and injected alone (○) or with a 1000-fold molar excess of α_2 M-methylamine (○). In each experiment, 1 μ g of radiolabelled protein was injected.

produced a derivative that cleared only via the α_2 M fast-form receptor (Fig. 6c). These data indicate that the carbethoxy derivatives of histidine are recognized by the acyl-LDL receptor system which recognizes acylated lysine residues.

In the next series of experiments, the clearance of native α_2 M modified with diethyl pyrocarbonate was studied (Fig. 7). It was predicted that the native α_2 M derivative would clear rapidly from the murine circulation because of histidine modification. However, the clearance was unaltered from that of native α_2 M. When this modified α_2 M was allowed to react with trypsin, the product was cleared by both the α_2 M fast-form receptor and the acyl-LDL pathway, as demonstrated by competition experiments similar to those described in Fig. 6. Competition for clearance was observed only when the histidine-modified 125 I- α_2 M fast-form was co-injected with a 1000-fold excess of α_2 M-methylamine and 2 mg of maleylated BSA. Neither agent alone was effective when injected as a competing ligand. Once again treatment with hydroxylamine reversed these effects. The hydroxylamine-treated preparation of 125 I- α_2 M fast-form was cleared only via the α_2 M fast-form receptor. It is concluded from these studies and those in Fig. 6 that diethyl pyrocarbonate modifies at least some histidine residues, which are not exposed to receptors until α_2 M undergoes slow-to-fast conformational change. Exposure of these modified residues permits clearance via the acyl-LDL pathway.

In the next series of experiments *cis*-DDP-modified α_2 M fast-form was derivatized with diethyl pyrocarbonate (1 mM) and studied in clearance experiments (Fig. 8). Treatment of α_2 M fast-form with these two reagents in

Fig. 8. Plasma clearance of radioiodinated α_2 M fast-form after reaction with *cis*-DPP and diethyl pyrocarbonate

The α_2 M fast-form preparation was allowed to react with 1.7 mM-*cis*-DPP and injected into mice before (○) and after reaction with diethyl pyrocarbonate for 5 min at the following concentrations: ■, 50 μ M; □, 250 μ M; ○, 1 mM. In one series of studies the α_2 M fast-form was treated with *cis*-DPP followed by 1 mM-diethyl pyrocarbonate and the preparation co-injected with 2 mg of maleylated BSA (●). In each experiment, 1 μ g of radiolabelled protein was injected.

this sequence yielded a derivative that was cleared rapidly from the murine circulation. When *cis*-DPP-treated α_2 M fast-form was allowed to react with 1 mM-diethyl pyrocarbonate and co-injected with 2 mg of maleylated BSA, clearance was completely blocked. These studies demonstrate that the *cis*-DPP-binding site responsible for blocking receptor recognition is separate from the diethyl pyrocarbonate sites that yield acyl-LDL-receptor-recognized species.

The final experiments were binding studies *in vitro* in which diethyl pyrocarbonate-modified α_2 M fast-form and underivatized α_2 M fast-form were used as competing ligands with 125 I- α_2 M-methylamine (Fig. 9). These studies demonstrated that histidine-modified α_2 M fast-form and the unmodified fast-form are recognized equally well by the α_2 M receptor. It is therefore highly unlikely that histidine residues are involved in the receptor recognition of α_2 M fast-form.

DISCUSSION

Macrophage binding studies *in vitro* were performed at 4 °C. The cells were metabolically inactive and endocytosis did not occur. Free *cis*-DPP did not affect the results of the cell binding experiments, indicating that the cytotoxicity of the compound is unimportant in this system. The results of the studies *in vitro* confirmed previous results obtained *in vivo* (Gonias & Pizzo, 1981a). Reaction with *cis*-DPP altered the receptor recognition site so that the protein no longer bound to cell surfaces. The protein modification was 95% complete after incubation with 1.7 mM-*cis*-DPP for 4 h. These studies are of particular interest, since *in vivo* metabolism of α_2 M fast-form occurs primarily via hepatocyte uptake, reflecting the greater mass of these

Fig. 9. Receptor binding by radiolabelled- α_2 M-methylamine *in vitro*: competition by α_2 M-methylamine or diethyl pyrocarbonate-treated α_2 M-methylamine

The α_2 M fast-form was allowed to react with 1 mM-diethyl pyrocarbonate before study. The conditions for the binding study are similar to those described in Fig. 1. The symbols are: competition of binding of 125 I- α_2 M-methylamine by underivatized α_2 M-methylamine (●) or by diethyl pyrocarbonate-treated α_2 M-methylamine (■). The K_1 was calculated as 0.36 mM in each case, essentially equivalent to the value for untreated α_2 M-methylamine listed in Table 1.

cells in liver as opposed to macrophage-like cells (Gliemann *et al.*, 1983; Feldman *et al.*, 1985b). It is concluded that loss of receptor recognition is a general phenomenon rather than a cell-specific event.

Diethyl dithiocarbamate is a potent chelator that probably forms a 2:1 molar complex with platinum (Gonias *et al.*, 1984). The ability of diethyl dithiocarbamate to reverse stable macromolecule-platinum bonds has been previously demonstrated (Gonias *et al.*, 1984). Reaction of *cis*-DPP-treated α_2 M-methylamine with diethyl dithiocarbamate resulted in total restoration of receptor recognition function. These data indicate that *cis*-DPP modification does not cause significant disruption of the tertiary structure of the α_2 M domain containing the receptor recognition site. The site remains intact with platinum bound to a critical amino acid.

H₂O₂ is a potent oxidant that reacts specifically with methionine and cysteine residues in proteins (Means & Feeney, 1971). The four cysteine residues present in α_2 M-methylamine are not involved in receptor recognition (Gonias & Pizzo, 1981a). α_2 M-methylamine that was allowed to react with H₂O₂ bound to cell membrane sites with greatly reduced affinity. This result suggests a role for methionine in receptor recognition. Structural alteration of the receptor recognition region was a second possible explanation of the data that needed to be considered. *cis*-DPP was utilized, therefore, to block specifically the reactive amino acid at the receptor recognition site. This pretreatment significantly protected α_2 M-methylamine from functional alteration caused by H₂O₂. There is now a large body of literature suggesting that *cis*-DPP reacts with methionine residues in the

absence of cysteine with a relatively high degree of specificity (Dickerson *et al.*, 1969; Howe-Grant & Lippard, 1980; Gonias *et al.*, 1984). The amino-acid-analysis data given here provide evidence that *cis*-DPP modification of methionine residues occurs and that this modification protects the receptor recognition site from destruction by H_2O_2 . On the basis of these data it is concluded that methionine is a critical amino acid in the primary sequence of the α_2M -receptor recognition site.

Since the amino acid analysis of H_2O_2 -treated α_2M -methylamine suggested that several histidine residues per subunit were oxidized, it was thought important to rule out the participation of histidine residues in receptor recognition. One argument against such participation was the observation that *cis*-DPP pretreatment of α_2M -methylamine did not protect these residues from destruction. More direct evidence addressing this question was then obtained by using the histidine-specific reagent diethyl pyrocarbonate. These studies demonstrated that a critical histidine residue is not at the α_2M -receptor recognition site. However, it was found that modification of about 13 histidine residues per α_2M subunit results in a derivative that is recognized by the same receptor that recognizes acyl-LDL or maleylated BSA (Goldstein *et al.*, 1979a,b; Imber *et al.*, 1982). To our knowledge, this is the first example of modification of an amino acid other than lysine resulting in protein recognition by this scavenger receptor. Of greater significance, the same degree of histidine modification occurs in both the slow and fast forms of α_2M , but conformational change is required before these modified histidine residues are receptor-recognized.

It is of interest that we have previously observed a similar conformational specific phenomenon with a much more primitive α -macroglobulin, namely that of the frog (Feldman & Pizzo, 1985). With this protein, conformational change exposes or properly orients carbohydrate such that it is receptor-recognized. We have recently proposed a model of α_2M structure (Feldman *et al.*, 1985a) that extends the 'trap' hypothesis of Barrett and his colleagues (Barrett & Starkey, 1973; Barrett *et al.*, 1979; Salvesen *et al.*, 1981). This model predicts that movement of two long arms per functional α_2M 'half-molecule' (Gonias & Pizzo, 1983c,d) exposes the α_2M -receptor recognition site as these arms swing on a hinge-like area located at the base of each half-molecule. Movement of the long arms occurs after cleavage of unique thioester bonds (Swenson & Howard, 1979; Sottrup-Jensen *et al.*, 1980; Salvesen *et al.*, 1981). There are four thioesters, one each per α_2M subunit. Our model predicts that these thioesters are at the base of the long arms of the α_2M half-molecule. Van Leuven *et al.* (1982) also have postulated that these thioesters may be in close proximity to the α_2M -receptor recognition site.

The present data concerning receptor recognition of modified histidines by the acyl-LDL receptor, as well as our studies with carbohydrate exposure in the frog homologue (Feldman & Pizzo, 1985), suggest that these carbohydrate chains and histidine residues are located near the hinge. As the hinge pivots, these moieties become more exposed to the solvent and are capable of engaging suitable receptors. Our model predicts that the α_2M -receptor recognition site is also located at the base of these long arms and is exposed when the hinge pivots during conformational change (Feldman *et al.*, 1985a). It

is suggested that the hinge may be viewed as a 'hot spot' from the standpoint of the function of α_2M .

Finally, it is possible to make a prediction as to the location the methionine residues involved in receptor recognition. Van Leuven and colleagues utilized monoclonal antibodies and chemical modification studies (Marynen *et al.*, 1982; Van Leuven *et al.*, 1982, 1983) and suggested that the α_2M -receptor recognition site is within the C-terminal third of the α_2M subunit. These investigators also suggested that there is a lysine residue at, or near, the site. Only six methionine residues are located towards the C-terminal side of the thioester in the α_2M subunits (Sottrup-Jensen *et al.*, 1984). Of these, Met-1291 is followed by Lys-1292; Met-1355 is six residues removed from Lys-1361; Met-1362 is adjacent to Lys-1361; and Met-1375 is preceded by Lys-1374. As a preliminary hypothesis, it is suggested that one of these methionine residues is at the α_2M -receptor recognition site.

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